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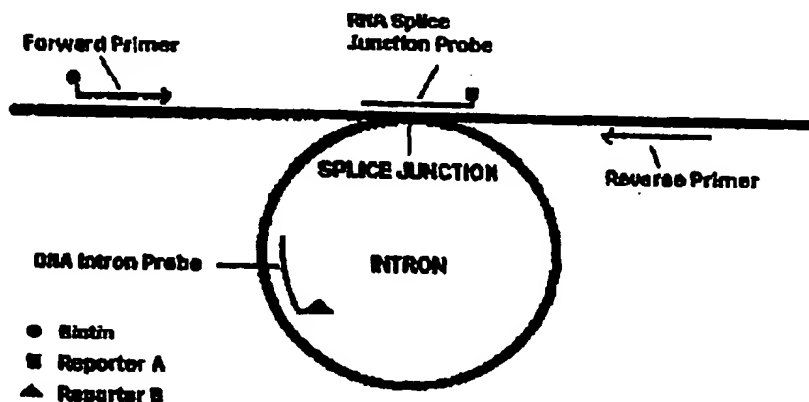
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(54) Title: QUANTITATION OF RNA TRANSCRIPTS USING GENOMIC DNA AS THE INTERNAL AMPLIFICATION COMPETITOR

## (57) Abstract

This invention describes a method for the quantitative monitoring of gene expression without either co-amplification of an added template or use of an endogenous constitutive transcript. The process involves a duplex amplification reaction in which a single set of primers is used to amplify both genomic DNA and expressed mRNA from the same gene sequence. These primers are targeted for sequences flanking the splice junction/intron

sequences for the mRNA/DNA, as shown in the figure. Use of these primers with any suitable nucleic acid amplification technology will yield mRNA and DNA amplimers which are distinguishable by length and sequence heterogeneity. These amplimers will also be present in the final amplification reaction in ratios which are dependent upon the ratios of the expressed mRNA to the DNA in the sample, thus allowing the quantitation of mRNA in a sample.



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## QUANTITATION OF RNA TRANSCRIPTS USING GENOMIC DNA AS THE INTERNAL AMPLIFICATION COMPETITOR

### FIELD OF INVENTION

5 This invention relates to the general fields of molecular biology, biochemistry, genetics and biological research, and specifically, relates to a method for the quantitative determination of relative amount and precise copy number of messenger RNA transcripts in biological samples. This method, thus, further relates to commercial applications in the areas of medical and biological research and medical diagnostics.

### 10 BACKGROUND AND PRIOR ART

The use of nucleic acid probe tests based on hybridization is hindered by a lack of sensitivity. Nucleic acid probe technology has been discovered for its value in the detection of various diseases, organisms or genetic defects, and thus, has developed quite rapidly. Current  
15 amplification techniques provide the sensitivity lacking in earlier assays and necessary to qualitatively determine the presence of minute quantities of nucleic acid. That is, they are sensitive enough to be able to detect a single copy of a specific nucleic acid sequence.

A number of methodologies are currently used for the  
20 measurement of gene expression. The most sensitive of these methodologies utilizes the polymerase chain reaction (PCR) technique, the details of which are provided in United States Patent No. 4,683,195, United States Patent No. 4,683,202, and United States Patent No. 4,965,188, all to Mullis *et al.*, all of which are specifically incorporated  
25 herein by reference. The details of PCR technology, thus, are not included herein. Recently, additional technologies for the amplification of nucleic acids have been described, most of which are based upon isothermal amplification strategies as opposed to the temperature cycling required for PCR. These strategies include, for example, Strand

Displacement Amplification (SDA)(United States Patent Nos. 5,455,166 and 5,457,027 both to Walker; Walker *et al.* (1992) PNAS 89:392; each of which is specifically incorporated herein by reference) and Nucleic Acid Sequence-Based Amplification (NASBA)(United States Patent No. 5,130,238 to Malek *et al.*; European Patent 525882 to Kievits *et al.*; both specifically incorporated herein by reference). Each of these amplification technologies are similar in that they employ the use of short, deoxyribonucleic acid primers to define the region of amplification, regardless of the enzymes or specific conditions used. This invention is concerned primarily with a novel amplification-primer methodology useful for the amplification and subsequent quantitation of nucleic acid using any amplification method, including but not limited to the aforementioned strategies. In particular, this application describes a method for the quantitation of RNA transcripts using genomic DNA as an amplification competitor.

Generally, amplification of DNA molecules utilizes a thermostable polymerase, for example, Taq polymerase, described in European Patent 258017, specifically incorporated herein by reference. Until recently, RNA amplification required a separate, additional step and the use of non-thermostable reverse transcriptase enzymes to generate a cDNA capable of being amplified by a thermostable DNA polymerase, such as Taq. The discovery of a recombinant thermostable enzyme (rTth) capable of coupling reverse transcription of the RNA with DNA amplification in a single enzyme: single reaction procedure greatly simplified and enhanced RNA amplification (see, Myers & Gelfand (1991) *Biochemistry* 30:7661-7666; United States Patent No. 5,407,800 to Gelfand and Myers, both specifically incorporated herein by reference).

Recombinant thermostable enzyme polymerase chain reaction (rTthPCR) is exquisitely sensitive. However, some notable difficulties are encountered when applying PCR to the quantitative measurement of gene

expression. All PCR-based methods for measuring gene expression have certain features in common: 1) the nucleic acids from a chosen sample must be extracted; and 2) the target sequence is amplified by PCR, allowing detection and quantitation of that sequence. The efficiencies of both the extraction step and the amplification step directly affect the outcome of the assay. Further, the detection step reveals how many copies of the target sequence have been made, but not how many copies were contained in the original sample. To determine the number of original copies, the efficiency of the nucleic acid extraction, as well as the efficiency of each PCR reaction must be known. An absolute quantitation of sample copy number is difficult using the current state of extraction technologies -- if not impossible. The best that can be achieved on a routine basis is quantifying changes in gene expression rate, rather than quantifying the exact numbers of copies of the target sequence contained in the sample. However, this quantification of gene expression rate changes can only be done if the efficiencies of the extraction and amplification processes are constant or can be controlled for. There are two basic types of control: the first is commonly known as exogenous control (Gilliland *et al.* (1990) *PCR Protocols*, Innis *et al.* ed., pp. 60-69, Academic Press; Wang *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:9717-9721, both specifically incorporated herein by reference), and the second, endogenous control (Dveksler *et al.* (1992) *PCR Methods and Applications* 6:283-285; Spanakis (1993) *Nucleic Acids Research* 21:3809-3819, both specifically incorporated herein by reference).

Exogenous control involves the use of an artificially introduced DNA or RNA molecule that is added, either to the extraction step or to the PCR step, in a known concentration. The concept of adding an exogenous nucleic acid at a known concentration in order to act as an internal standard for quantitation was introduced by Chelly *et al.* (1988) *Nature* 333:858-860, specifically incorporated herein by reference. This

approach was improved following the realization that PCR amplification efficiency is dependent upon the primer sequences (WO 91/02817 to Wang *et al.*, specifically incorporated herein by reference). Therefore, utilizing a control fragment that is amplified with the same primers as the target sequence more accurately reflects target sequence amplification efficiency relative to the internal standard (see, for example, WO 93/02215 to Fox and Griffiths; WO 92/11273 to McCallum *et al.*; United States Patent No. 5,213,961 to Bunn *et al.*; and, United States Patent No. 5,219,727 to Wang *et al.*, all of which are specifically incorporated herein by reference). Similar strategies have proven effective for quantitative measurement of nucleic acids utilizing isothermal amplification reactions such as NASBA (Kievits *et al.*, *supra*) or SDA (Walker, *supra*).

The use of an endogenous control regulates variations in extraction efficiency. Control choice is important in that several requirements must be met in order for it to work. The first requirement is that the copy number of the control must remain constant; the second, the control must amplify with similar efficiency to the sequence being monitored. Several constitutively expressed genes have been considered as control candidates, since the expression of these genes is relatively constant over a variety of conditions. The most common of these are the  $\beta$ -actin gene, the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), and the 16S ribosomal RNA gene. While these genes are considered to be constitutively expressed, they are not ideal for the above-discussed purpose. The first problem is that under some conditions the expression level of these genes varies (Spanakis (1993) *supra*; Dveksler *et al.* (1992) *supra*) possibly invalidating them as controls. Further, these genes necessitate using a different set of PCR primers from any other gene being assayed, possibly leading to differences in PCR efficiency, and thus, invalidating them as controls. Zenilman *et al.* ((1995) *Analytical Biochemistry* 224:339-346, specifically incorporated herein by reference)

attempted to design a competitive polymerase chain reaction method that altered the cDNA generated during reverse transcription using a composite primer to introduce a common intron primer sequence into the cDNA, such that both genomic DNA and cDNA could then be amplified with the same primer set. That method requires careful design of the composite primers so that, following reverse transcription, the melting temperature ( $T_m$ ) differential between the composite and DNA primers is adjusted to prevent the composite primer from binding to the genomic DNA. It further requires separate steps for reverse transcription and DNA amplification, and uses a cumbersome, impractical radioactive detection methodology.

The detection of amplified products following quantitative PCR must provide a method of distinguishing the added control standard from the target nucleic acid sequence. Endogenous controls include, for example, radioactive probes specific to the control sequence, wherein the control and target detection is split into separate detection reactions for each amplification tube. Exogenous standards have been designed so as to be distinguishable by size of the amplicon as visualized on an agarose gel (Scadden *et al.* (1992) *J. Infect. Dis.* 165:1119-1123; Piatak *et al.* (1993) *Biotechniques* 14:70-80, both specifically incorporated herein by reference) or by introducing an internal restriction site through mutagenesis, wherein the restriction fragments are again detected on an agarose gel (Becker-Andre and Hahlbrock (1989) *Nucleic Acid Res.* 17:9437-9446; Steiger *et al.* (1991) *J. Virol. Methods* 34:149-160, both specifically incorporated herein by reference). All of these methods employ a relatively insensitive and imprecise detection method. More recently developed methods utilize chemiluminescent or enzyme-linked immunosorbent assay (ELISA) based detection that more precisely measure amplified product (Mulder *et al.* (1994) *J. Clin. Microbiol.* 32:292-300, specifically incorporated herein by reference).

It is preferable to utilize detection methods involving a homogeneous assay, wherein detection occurs concomitantly with amplification. This is possible utilizing the nuclease assay methodology described in United States Patent No. 5,210,015 to Gelfand *et al.*, specifically incorporated herein by reference. The preferred probes for the nuclease assay, those probes having the greatest specificity, are described by Marmaro *et al.* (USSN 08/340,558; USSN 08/559,405; and USSN 08/558,303, the specifications of which are specifically incorporated herein by reference). These preferred probes, designated "TaqMan" probes and developed by the Perkin Elmer Corp. (Foster City, CA), utilize energy transfer fluorescence methods, wherein the probes are self-quenched until cleaved during the PCR nuclease assay. Cleavage during PCR generates an increased fluorescence signal. Furthermore, several reporter dyes can be monitored in the same reaction.

The characteristics of an ideal quantitation control include: its presence in unchanging concentrations in the cell; it possesses the same PCR efficiency as the system being assayed; and, it is easily distinguishable from the system being assayed. It is further desirable that the control is a natural component of the biological specimen making it unnecessary to synthesize and externally add an artificial standard, and the control must accurately accommodate fluctuations both in nucleic acid extraction as well as amplification efficiencies. Finally, the control must be accurately distinguishable from the target, preferably by an homogeneous assay of such specificity so as to have no cross reactivity that alters the ratio of the two. The quantitation methodology disclosed herein meets these requirements by integrating existing technologies with novel amplification and primer design techniques, as disclosed herein.



## SUMMARY OF THE INVENTION

This invention is based on a novel concept for a method of quantitatively measuring the level of specific gene expression in biological samples. More specifically, a method for the quantitative determination of RNA transcripts using genomic DNA as an internal amplification competitor. That is, an amplification method using genomic DNA as the endogenous quantitation standard. Furthermore, the present invention encompasses a novel method for designing amplification primers such that they exploit sequence differences and/or length differences that occur with design strategies, and therefore, any method wherein primers are employed to define the amplification region may be used in concert herewith. The instant invention is defined by an extraction step, a designing step, a hybridization and amplification step and a determination step.

The present invention is applicable to all amplification methods, in which the amplification of both DNA and RNA is provided in a competitive reaction. Moreover, the present invention is applicable to any transcribed gene, wherein both the RNA and DNA sequences are characterized, but where there also exists some sequence heterogeneity between the RNA and DNA sequences. The present invention is useful for reliable, quantitation of specific gene transcription --determining changes in ratios or copy numbers of RNA per cell -- but is not intended to be limited to this use.

In an embodiment of this invention, a nucleic acid sample is obtained, primer and probe sequences designed, hybridization and amplification performed and relative gene expression determined by relative quantitation of RNA and DNA amplimers.

In another embodiment of this invention, genomic DNA and mRNA are amplified by PCR using a single set of primers, said primers designed such that they are located within two exons flanking an intron of

a certain size, as dictated by the method of amplification, and two probes fluorescently tagged, one of which is specifically hybridized to the mRNA splice junction, and the other hybridized to a sequence contained only in the intron of the same gene. For example, in certain methods of amplification, an intron that is too large in size compromises the amplification efficiency. This embodiment enables the application of the instant invention in such cases through the modification of primer design strategies. Gene expression is thus determined through multiplex componenting the ratio of RNA (splice junction probe) to DNA (intron probe) signals.

The invention described herein provides for a duplex amplification reaction allowing competitive PCR quantitation. PCR primers designed to be within two exons flanking an intron facilitate DNA and RNA being amplified by PCR using a single set of primers. The DNA amplimer is longer than the mRNA amplimer by the length of the intron. The DNA amplimer is further distinguishable from the mRNA amplimer by sequence heterogeneity. That is, the DNA amplimer contains the intron sequence, while the RNA amplimer does not. The mRNA amplimer contains the continuous splice junction sequence, while the DNA amplimer does not. The PCR products of the DNA are thus distinguishable from those of the RNA by any method that capitalizes on the above-described differences. For example, by using "TaqMan" (5'-nuclease) probes in which one probe with a certain reporter dye binds to an intron sequence and another with a different reporter dye binds to the splice junction sequence in a PCR amplification reaction, the ratios of DNA amplimer to RNA amplimer are determined. The sequence specificity of these TaqMan probes prohibits signal cross-talk between DNA and RNA amplimer measurements. Thus, the ratio of the mRNA signal to that of the DNA accurately reflects the ratio of mRNA to DNA in the original sample. This method of measurement can be applied to

accurately and conveniently monitor changes in gene expression independent of extraction and amplification efficiency, since DNA signal always serves as the endogenous quantitation standard.

5 The instant invention relates generally to quantitative gene expression determination. This invention relies upon principles of hybridization primer based nucleic acid amplification, including, for example, PCR, SDA and NASBA. The process and method according to the present invention is suitable for the quantitation of all characterized genes for which some degree of mRNA processing results in sequence  
10 heterogeneity between the DNA and the mRNA, such as intron excision and splicing. The sensitivity and accuracy of the methods disclosed herein are improved in relation to current technologies, offering the possibility of simplicity, reliability and specificity.

15 Other features and advantages of the instant invention will become apparent from the following detailed description, taken in conjunction with the accompanying figures, that illustrate by way of example, the principles of the instant invention.

#### **BRIEF DESCRIPTION OF THE FIGURES**

20 Figure 1 is a schematic representation of the intron/exon structure for the IL-2 gene.

Figure 2 is a schematic representation of the intron/exon structure for the GAPDH gene.

Figure 3 depicts the strategy for primer and probe design.

Figure 4 shows an agarose gel electrophoretogram exhibiting DNA and RNA banding patterns following a PCR titration of mRNA to a constant DNA concentration.

5        Figure 5 shows the RNA IL-2 probe signal using an RNA probe only in amplification reactions at various time intervals following induction of Jurkat cells.

Figure 6 is the DNA IL-2 probe signal using a DNA probe only in amplification reactions at various time intervals following induction of Jurkat cells.

10        Figure 7 displays the data of Figures 5 and 6 as the ratio of the RNA to DNA ratios at each time point.

Figure 8 depicts the IL-2 RNA to DNA ratios observed when both probes are present in the same amplification reactions at various time intervals following induction of Jurkat cells.

15        Figure 9 depicts the GAPDH RNA to DNA ratios observed when both probes are present in the same amplification reactions at various time intervals following induction of Jurkat cells.

20        Figure 10 depicts the IL-2 protein detectable by means of an ELISA reaction at various time intervals following induction of Jurkat cells.

Figure 11 represents a generic hybridization-capture probe scheme.

Figure 12 illustrates a common primer and two downstream primers used for amplification and discrimination.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

5 It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

The instant invention describes a method for quantitatively measuring the level of transcription of specific genes in biological specimens. Gene expression that may be determined according to the  
10 present invention is that of any transcribed gene in which both the RNA and DNA sequences have been characterized and which contain sequence heterogeneity between the DNA and the mRNA. By way of illustration, included herein is the exon/intron characteristics of the human IL-2 gene (Figure 1) or GAPDH gene (Figure 2).

15 The general principles and conditions for amplification of nucleic acids are well known in the art. In the broadest conception of the invention, the present invention specifies the duplex amplification, preferably with a single set of primers, capable of amplifying both genomic DNA and expressed mRNA from the same gene sequence,  
20 allowing competitive PCR quantitation based upon changes in ratio of mRNA to genomic DNA. In the simplest embodiments of the invention, relative copy numbers of mRNA and DNA are determined by separating the amplimers by size and measuring or estimating their relative amounts. Since the DNA amplimer is longer than the mRNA amplimer, gel  
25 electrophoresis or other size-discriminating chromatography separates them, Figure 4. Each amplimer is visualized and quantified by autoradiography, radiographic counting, densitometry, or any such technique. In other embodiments of the invention, relative copy numbers

of the mRNA and DNA are determined by using two hybridization/detection probes: one, preferably, specific to the intron sequences of the DNA; and the other, preferably, exclusively hybridizing to only mRNA at the splice junction (see, Figure 11). These probes are then used to allow measurements of the DNA to mRNA amplimer ratios. Examples are two such probes designed for use with the IL-2 detection system. The sequences of interleukin 2 capture probe sequences for use with the primer sequences disclosed in Table I are provided in Table II. Selective label reporters A and B can be fluorescent label, antibody hapten, electrochemiluminescence label, bioluminescence label, chemiluminescence label or any enzyme label standardly used for ELISA assays. Selective labeling of the detection probes allows their use in a variety of detection technologies, for example, fluorescence detection, electro-chemiluminescence detection, chemiluminescence detection, bioluminescence detection and enzyme-linked immunosorbent detection (ELISA).

In an alternate embodiment, only one common hybridization primer for amplification is used. In place of the second amplification primer and two distinct detection probes, two distinct primer/detection probes are used, Figure 12. In this way, the DNA amplimer is defined by the common primer with the second amplification primer binding to the intron. The mRNA amplimer is defined by the common primer and its second amplification primer binding to the splice junction sequence. The DNA and mRNA specific primers act as combination amplification primers and detection probes and are labeled for this purpose. This embodiment is most useful when only very large introns are present in the DNA for the gene. Flanking much larger introns with two common primers can be problematic with current amplification technologies. Removing the requirement for two common primers allows freedom in determining the amplimer length, independent of intron size.

In the most preferred embodiment of the invention, the level of transcription of specific gene is quantitatively measured by: a) nucleic acid extraction; b) primer and probe sequence design; c) polymerase chain reaction; and d) quantitative gene expression determination.

5 One skilled in the art recognizes that quantitation, as disclosed herein, may be performed in a broad range of biological samples. Such samples include, for example, biological samples derived from agriculture sources, bacterial and viral sources, and from human or other animal sources, as well as other samples such as waste or drinking water, 10 agricultural products, processed foodstuff and air. More specifically, samples include, for example, blood, stool, sputum, mucus, serum, urine, saliva, teardrop, biopsy samples, histological tissue samples, tissue culture product, an agricultural product, waste or drinking water, foodstuff and air. The present invention is useful for the quantitation of RNA 15 transcripts indicative of specific gene expression in any sample containing nucleic acid.

Various terms are used in this specification, for which it may be helpful to have definitions. These are provided herein, and should be borne in mind when these terms are used in the following examples and 20 throughout the instant application.

As used herein, the term "amplification" refers to a template-dependent process by which multiple copies of nucleic acid sequences are formed, resulting in an increase in the concentration of a nucleic acid sequence relative to its initial concentration.

25 As used in this invention, a "template-dependent process" is defined as a process that involves the template-dependent extension of a primer molecule. A template-dependent extension refers to nucleic acid synthesis of RNA or DNA wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the rules of complementary base 30 pairing of the target nucleic acid and the primers.

A "primer" molecule refers to a nucleic acid sequence, complementary to a known portion of the target sequence/control sequence, necessary to initiate synthesis by DNA polymerase. A short primer sequence base-paired to a specific site, for example, an intron, on a longer nucleic acid can initiate polymerization from that site. As used  
5 in this invention, a "common intron primer" is defined as a primer sequence that is identical for different targets, i.e., they have the same primer sequences in common. Further, a "composite primer" as used herein, refers to a primer consisting of a hybrid with a portion of the  
10 sequence complementary to the target, and the remainder non-complimentary. During the PCR reaction the polymerase copies through the non-complimentary sequence resulting in its incorporation into the target. "Competitive PCR" refers to amplification reactions wherein the control fragment and the target utilize the same primer sequences  
15 resulting in competition of the primers for the target and the control fragment. "Target nucleic acid sequence" refers to the nucleic acid molecule that is to be amplified. The target molecule can be present in a purified, partially purified or unpurified state in the sample.

"Control fragment" is defined as a nucleic acid sequence utilized as  
20 a standard in quantitation. "Internal fragment" is the nucleic acid present in each individual PCR reaction that can be utilized as a control.

As used herein, "amplification competitor" refers to a nucleic acid containing the same primer recognition sequences as the target nucleic acid molecule.

25 "Amplimer" is a term of art, known to and understood by the skilled artisan, as referencing the nucleic acid amplification product.

"Constitutive gene expression" means that the genes are expressed in all cells at relatively constant levels and are not regulated or induced.

This application used "signal cross-talk" to refer to the degree of  
30 specificity as measured by the detection signal of two different probes.



The present invention is embodied in a method for the quantitative determination of gene expression, particularly useful for small tissue samples. Regardless of the specific application of the instant invention, the methodology details are calculated according to protocols well known in the art as well as those disclosed herein. Further, the refinement of said necessary calculations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them without undue experimentation.

One embodiment of the present invention is a method of quantitatively measuring the level of transcription of specific genes in biological specimens, said method comprised of the following steps :

a) extracting from any biological specimen the nucleic acid in such a manner such that both the DNA and RNA are obtained in a consistent yield ratio;

b) designing primer and probe sequence for performing amplification reaction in such a manner that both the messenger RNA and DNA are amplified by means of the same primer recognition sequences;

c) performing PCR utilizing a single tube one step reaction wherein the above primers and probes are included together with recombinant reverse transcriptase enzyme in such a manner as to generate distinguishable fluorescent signals specific to RNA or DNA by means of a homogeneous nuclease reaction; and

d) determining relative gene expression through multiplex componenting the ratio of the RNA to DNA fluorescent signals, or determining absolute RNA copy number relative to a standard curve derived by the ratio of known concentrations of purified RNA and DNA for the gene.

Primer and probe sequences are designed specifically for performing amplification reaction in such a manner that both the messenger RNA and DNA are amplified by means of the same primer

recognition sequences. That is, said primers are designed so as to be located within two exons flanking an intron of a size consistent with the amplification method capability. In such cases where the introns for a target gene all exceed the size readily amplified by the amplification technology of choice, one common primer for both mRNA and DNA is placed in an exon adjacent to the intron. The second primer for DNA amplification is located in the intron sequence and the second primer for the mRNA amplification is complimentary to the splice junction sequence for that intron. In this case, the second primers simultaneously serve as detection probes. In all cases, the probes are labeled with reporter molecules or haptens to facilitate their detection. An illustrative example of the method is the use of PCR as the amplification technique and TaqMan probes for detection. The TaqMan probes are bifunctionally tagged with fluorophores so as to be self-quenching. The two probes are further designed so that one probe with reporter fluorescent tag 'A' specifically hybridizes only within the DNA of the particular gene intron, while the second probe with reporter fluorescent tag 'B' specifically hybridizes only to the splice junction of the messenger RNA for the same gene. Probe and primer design is illustrated in Figure 3.

The method disclosed and taught herein is applicable not only to the preferred PCR-based embodiment, as discussed above, but also to any and all other amplification methods, wherein the amplification of both DNA and RNA is provided in a competitive reaction and/or detection probes are designed to measure DNA intron sequences relative to mRNA sequences at the splice junction.

Another embodiment, for example, is the use of gap filling ligase chain reactions (see, United States Patent No. 5,427,930 to Birkenmeyer *et al.*, specifically incorporated herein by reference). In a ligase application, one set of ligation probes is designed to be specific to DNA intron sequences, while a second duplex set in the same reaction is

designed to be specific to the mRNA splice junction. Following ligase chain reaction amplification with all probes included within one reaction tube, the detection step is split into two tubes each developed for different reporter haptens in the established LCx format.

5           Due to its simplicity, specificity for transcription, and high sensitivity, this invention enables transcription to be more reliably and readily measured. In particular, this invention is applicable to small tissue samples, for example, fine needle biopsies of tumors or  
10           transplanted organs, amniotic fluid sampling, atherosclerotic tissue, and other biological specimens previously requiring invasive procedures to obtain adequate material for measuring specific gene transcription. The instant invention is readily amenable to commercialization in the biology, biomedical research, and clinical diagnostic arenas, for example, in the form of kits including primer sets and corresponding detection probes for  
15           the gene of interest.

          Those skilled in the art readily recognize the present invention is broadly applicable to any transcribed gene in which both the RNA and DNA sequences have been characterized and in which there is sequence heterogeneity between the RNA and the DNA. The following examples  
20           serve to explain and illustrate the present invention. Said examples are not to be construed as limiting of the invention in anyway. Various modifications are possible within the scope of the invention.

#### **Example 1 PCR Probe and Primer Design**

          Primers and probes are designed as outlined in Figure 3 and Table  
25           I. The primers amplify both RNA and DNA in the same reaction by means of the same primer sequences. The probes are "TaqMan" probes specific to the DNA intron or the splice junction of the RNA. PCR primer and "TaqMan" probes are designed for both the Human GAPDH gene and the Human IL-2 genes (see, Figures 1 and 2). PCR primers

flanking the one, small IL-2 intron were designed and subsequently purchased from Operon Technologies (Alameda, CA). Two "TaqMan" probes, one for binding to the intron for DNA detection and the other for binding across the splice junction for RNA detection, were designed and subsequently ordered from Applied Biosystems Division of Perkin Elmer (Foster City, CA)(see, Figure 1). The DNA-specific "TaqMan" probe was made with a different reporter fluorophore from that used in the RNA probe. A similar set of PCR primers and "TaqMan" probes was designed for use with the fourth intron in the GAPDH gene (see, Figure 2).

### **Example 2 Isolation of Nucleic Acid**

A commercial rapid preparation, "QIAamp HCV", is utilized for the isolation and extraction of total nucleic acid per the manufacturer's instructions (Qiagen Inc., Chatsworth, CA). The method disclosed herein is compatible with any nucleic acid extraction methodology, wherein both DNA and RNA are purified.

### **Example 3 Confirmation of Probe Specificity**

The first set of experiments performed with the components of this assay were designed to confirm the lack of cross-talk between the DNA and RNA "TaqMan" probes using RAJI cell DNA and RNA and the GAPDH gene probes. The results of these experiments confirm a lack of cross-talk, but only after retreating the pure RNA preparation with preparations from DNA contamination or vice versa. Simplex PCR (only one "TaqMan" probe in a reaction) using purified DNA and RNA shows signal generation using RAJI cell DNA and RNA, respectively. Also, cross-talk is observed between the GAPDH gene DNA probe and the RAJI cell RNA preparation; cross-talk is not observed between the RNA probe and the RAJI cell DNA preparation. Further treatment of the RNA

preparation with additional DNases eliminates this cross-talk. Similar experiments with the IL-2 "TaqMan" probes give similar results, not shown here.

**Example 4 GAPDH Constitutive Transcription / IL-2 Induction**

5 Jurkat cells, American Type Culture Collection No. T1B-152 clone E6-1 (ATCC E6-1), are grown to the desired cell density and split up into 1.0 ml cultures. Mitogen is added to these in 0X, 1X, 5X and 10X concentrations and the cultures returned to the incubator and harvested at the following times: 2 hours, 4 hours, 8 hours, 24 hours, 48 hours and  
10 72 hours subsequent to mitogen addition. The total nucleic acid is extracted and purified using "QIAamp" columns and stored frozen, at -20 °C (Qiagen Inc., Chatsworth, CA). "TaqMan" PCR with nucleic acid preparations from Jurkat cells give signal for both the DNA and RNA GAPDH, and the IL-2 DNA probes.

15 IL-2 induction is performed using Jurkat cells grown to a density of  $2 \times 10^6$  cells per milliliter with phytohemagglutinin and phorbol myristate acetate as mitogens (1.0  $\mu\text{g}/\mu\text{L}$  and 50  $\text{ng}/\mu\text{L}$ , respectively). The cells are harvested at various time points following mitogen addition with a no-mitogen control at each time point. Total nucleic acid is  
20 extracted using a "QIAamp HCV" kit (Qiagen Inc., Chatsworth, CA). For both RNA and DNA amplification via PCR, the Perkin Elmer "GeneAmp EZ" rTth RNA PCR kit is used (Perkin Elmer Corp., Foster City, CA). PCR conditions are as follows: each 50  $\mu\text{L}$  reaction is done in 1X EZ RT buffer; 25 mM  $\text{Mg}(\text{OAc})_2$ ; 300  $\mu\text{M}$  each dNTP; 500 nM  
25 each PCR primer; 100 nM each "TaqMan" probe; and 0.1 units/ $\mu\text{L}$  rTth DNA polymerase. Nucleic acid from each cell extraction is used as template in a concentration of 1 to 5  $\mu\text{g}/50 \mu\text{L}$  PCR reaction. Cycling conditions are: 15 minutes at 60 °C for RT; followed by 50 cycles of 15 seconds at 94 °C melt and 1.0 minute at 55 °C for anneal/extend. After

PCR the samples are cooled to 4 °C and transferred to a Perkin-Elmer "Hi-White" microtiter plate. Fluorescence detection is done using a Perkin-Elmer LS-50B fluorescence spectrometer with microplate reader attachment (Perkin Elmer Corp., Foster City, CA ). Multi-componenting analysis and all subsequent calculations are performed by computer, using Microsoft "Excel" (Microsoft Corp.). The ratio of the RNA signal divided by the DNA signal for each sample is plotted against the amount of time following mitogen addition. To check for cross-talk between the DNA and RNA "TaqMan" probes, purified DNA and RNA preparations from RAJI cell lines are used in simplex PCR. The RAJI nucleic acid preparations were the kind gift of Paul Wyatt of the Applied Biosystems Division of the Perkin Elmer Corporation.

Signal from the IL-2 RNA probe is only seen from cells grown in the presence of the mitogen. For the IL-2 assays, the first PCR reactions are simplex reactions having either the RNA probe or the DNA probe. The signal from each probe is plotted against time for each level of mitogen addition (see, Figures 5 and 6). From this data, the RNA signals at each time point are ratioed against the DNA signals from the same points and plotted in Figure 7. Following this, a similar assay is set up in which both DNA and RNA probes are present in each reaction. The plots of the RNA/DNA ratios against time are shown in Figure 8.

Multiplex PCR with both GAPDH probes is carried out with all of the same samples, as indicated in the above-disclosed simplex PCR, and the results are plotted in a similar fashion as well (see, Figure 9). From each of the figures presented herein, it is apparent that the IL-2 mRNA is expressed only in the presence of mitogen, and is expressed early. The amount of IL-2 DNA is relatively constant, showing some variation at the early time points. This may be due to differences in cell density following mitogen addition. The effect of mitogen on cell growth and density is discussed below. Examination of the ratio of RNA to DNA,

both in the graph showing the ratios of the simplex data (Figure 7) and the graph of the duplex data (Figure 8), clearly shows the increase if IL-2 mRNA following induction. The specificity of the IL-2 induction was verified by assay of all cell supernates for IL-2 protein using the "Predicta" IL-2 kit (Genzyme Diagnostics, Cambridge, MA). The amount of GAPDH mRNA relative to DNA is largely constant. It is observed, from the simplex IL-2 DNA data (Figure 10), that clearly the absolute amount of DNA in the cells that did not have mitogen added is greater than that amount of DNA in the cells receiving mitogen. Close examination of the cell cultures shows that in the presence of mitogen there is significant cell-mortality, up to 50%. The DNA data show that this system detects absolute changes in nucleic acid concentration when the DNA and RNA data are examined alone. In spite of the large apparent difference in absolute amounts of nucleic acid present in the various samples, the ratio of the RNA signal to the DNA signal is insensitive to absolute amounts of nucleic acid, but is sensitive to changes in the ratio of mRNA to DNA. Since both nucleic acid extraction procedures and PCR can vary in efficiency, a system that is insensitive to absolute levels of nucleic acid and sensitive to changes in the mRNA to DNA ratio for a given gene is of great value. This simple, yet powerful, approach clearly accomplishes this goal and promises to have great utility for gene expression studies in a large variety of systems.

#### **Example 5 Size Discrimination Amplimer Detection**

Any technique that detects and/or quantifies nucleic acids based upon size or sequence discrimination, for example gel electrophoresis, hybridization capture and TaqMan PCR, respectively, is suitable for amplimer detection with this invention. The simplest such methodology is agarose gel electrophoresis. This technique is dependent upon the tendency for nucleic acids, which have a net negative charge in solution,

to migrate towards the positive pole in an electric field. That is to say, the resulting amplimers from the reaction migrate towards the positive pole. The gel matrix physically retards the flow of the nucleic acid, therefore, shorter molecules migrate more quickly than longer ones.

5 Hence, separation of a mixture of nucleic acids based upon size occurs and the various sizes of nucleic acids form bands of material comprised of similarly sized molecules. DNA amplimers formed by the process comprising this invention are longer than the RNA amplimers formed, and thus, migrate more slowly in this type of gel electrophoresis. A  
10 typical result is two discreet bands of material; the DNA amplimers and the mRNA amplimers.

A model PCR experiment using the primers for the GAPDH gene, intron 4, was performed. In this experiment, the amount of input purified genomic DNA is used for the DNA template (provided by Dr. Paul  
15 Wyatt). Purified human poly A mRNA, assayed for GAPDH mRNA, is used for the mRNA template. This often used preparation of messenger RNAs is purified by virtue of the presence of long homopolymeric runs of Adenosine bases added to mRNAs in eukaryotic organisms. The amount of GAPDH mRNA and genomic GAPDH DNA in each template  
20 had been carefully quantified by limiting dilution PCR. Four PCR reactions are set up in which the GAPDH DNA is held constant at 25 copies and the GAPDH mRNA is varied at 5, 50, 500 and 5000 copies of input template. After 40 cycles of PCR amplification (reaction conditions identical to those described *supra*, sans TaqMan probe), aliquots of each  
25 reaction are placed in loading buffer and run on a 3% agarose gel at 125 volts constant current for 15 minutes, standard electrophoresis conditions. The agarose gel is prepared with ethidium bromide (EtBr) for band visualization. The resultant gel is shown in Figure 4 in which the DNA band is clearly distinguishable from the RNA band. The relative band  
30 intensities exhibited in the gel clearly reflect the ratio of input RNA to



DNA changes. While simple visualization of the amplimers in gel electrophoretograms is somewhat crude, more precise gel-electrophoresis quantitation methodologies may be used to actually quantify the intensities of the bands. The instant gel serves to illustrate the ease with which the DNA amplimers are distinguished from the RNA amplimers on the basis of size, while the above TaqMan fluorescent signal example illustrates discrimination by sequence.

While the above description contains many specificities, these specificities should not be construed as limitations on the scope of the invention, but rather exemplification of the preferred embodiment thereof. That is to say, the foregoing description of the invention is exemplary for purposes of illustration and explanation. Without departing from the spirit and scope of this invention, one skilled in the art can make various changes and modifications to the invention to adapt it to various usages and conditions. As such, these changes and modifications are properly, equitably, and intended to be within the full range of equivalence of the following claims. Thus, the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples provided herein.

TABLE I

INTERLEUKIN 2 PRIMER AND PROBE SEQUENCES

## PCR PRIMERS

FORWARD PRIMER (IL-2 Forward ) bp:313-329

5' -GTCTTGCACTGCACTAA SEQ ID NO:1

REVERSE PRIMER (IL-2 Reverse)

5' -TAAATGTGAGCATCCTG SEQ ID NO:2

RNA TAQMAN PROBE (SPLICE-JUNCTION PROBE) bp-mRNA:203-233

5' -R-TTGGGATTCTTGTAATTATTAATTCCATT-Q SEQ ID NO:3

10 DNA TAQMAN PROBE (INTRON PROBE)

5' -R-TTGCACTTGTCACAAACAGTGACCT-Q SEQ ID NO:4

GAPDH PRIMER AND PROBE SEQUENCES

## INTRON 2

## PCR PRIMERS

15 FORWARD GFP12 bp:2457-2474

5' -GTTATTGGGCGCCTGGTC SEQ ID NO:5

REVERSE GRP12 bp:3399-3417

5' -CGCTCCTGGAAGATGGTGA SEQ ID NO:6

RNA SPLICE JUNCTION PROBE GDRTPIN2 bp-mRNA:174-203

20 5' -R-TGACCTCAACTACATGGTTTACATGTTCCA-Q SEQ ID NO:7

DNA INTRON PROBE GDTPI2 bp:3274-3300

5' -R-CAGCCCCTTCATACCCTCACGTATTCC-Q SEQ ID NO:8

## INTRON 4

## PCR PRIMERS

25 FORWARD GFP14 bp:3575-3593

5' -TGCGCTGAGTACGTCGTG SEQ ID NO:9

REVERSE GRP14 bp:3770-3789

5' -ACATGGGGGCATCAGCAGAG SEQ ID NO:10

RNA SPLICE JUNCTION PROBE GDTPI4 bp-mRNA:373-396

30 5' -R-ATGAGAAAGGCTGGGGCTCATTG-Q SEQ ID NO:11

DNA INTRON PROBE GRTPIN4 bp:3662-3696

5' -R-AGCTGACTCAGCCCTGCAAAGGCA-Q SEQ ID NO:12

\*R=Reporter Dye

\*Q=Quencher Dye

TABLE II

INTERLEUKIN 2 CAPTURE PROBE SEQUENCES

	mRNA SPLICE JUNCTION CAPTURE PROBE bp:182-205	
	5'-TTATTAATTCCATTCAAAATCATC-Reporter A	SEQ ID NO:13
5	INTRON SEQUENCE CAPTURE PROBE bp:488-511	
	5'-TGTTATTAAGAAATGATCTCCAGC-Reporter B	SEQ ID NO:14

Reporter A and B can be:

1. Fluorescent Label, such as:
  - Fluorescein
  - 10 -Fluorescein Isothiocyanate (FITC)
  - Any fluorophore having an emission spectrum allowing spectral discrimination between reporter A and reporter B, for example, Fluorescein and Texan Red pair
2. Antibody hapten, such as:
  - 15 -Di-Nitrophenol (DNP)
  - Digoxigenin
  - Any hapten for which a specific antibody is available
3. Electrochemiluminescence label, such as:
  - 20 -Ruthenium label
  - Any electrochemiluminescent label
4. Bioluminescence label, such as:
  - Aequorin
  - Any bioluminescent label
5. Chemiluminescence label, such as:
  - 25 -Lumiphos
  - Any chemiluminescent label
6. Enzyme label, such as:
  - Any used for standard ELISA assays

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: John C. Gerdes  
Jeffrey M. Marmaro
- 5 (ii) TITLE OF INVENTION: QUANTITATION OF RNA  
TRANSCRIPTS USING GENOMIC  
DNA AS THE INTERNAL  
AMPLIFICATION COMPETITOR
- (iii) NUMBER OF SEQUENCES: 14
- 10 (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Julie L. Bernard  
(B) STREET: 9000 E. Inspiration Drive  
(C) CITY: Parker  
(D) STATE: Colorado  
15 (E) COUNTRY: USA  
(F) ZIP: 80134-8535
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Diskette, 3.5 inch  
(B) COMPUTER: IBM PC Compatible  
20 (C) OPERATING SYSTEM: MS-DOS  
(D) SOFTWARE: WordPerfect 6.0
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE: 2 May 1996  
25 (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 60/016,710  
(B) FILING DATE: 2 May 1996
- 30 (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Julie L. Bernard  
(B) REGISTRATION NUMBER: 36,450  
(C) REFERENCE/DOCKET NUMBER: IAD-3PCT
- (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: 303 841 7472  
35 (B) TELEFAX: 303 840 1567

- 5 (2) INFORMATION FOR SEQ ID NO:1:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 17 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
GTCTTGCA TT GCACTAA 17
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(A) LENGTH: 17 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
15 (ix) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
TAAATGTGAG CATCCTG 17
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(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ix) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
TTGGGATTCT TGTAATTATT AATTCCATT 29
- 25 (2) INFORMATION FOR SEQ ID NO:4:  
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(A) LENGTH: 26 bases  
(B) TYPE: nucleic acid  
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(B) TYPE: nucleic acid  
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(D) TOPOLOGY: linear  
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(A) LENGTH: 27 bases  
(B) TYPE: nucleic acid  
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(D) TOPOLOGY: linear  
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30 (D) TOPOLOGY: linear  
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TGGCGCTGAG TACGTCGTG 19
- 35 (2) INFORMATION FOR SEQ ID NO:10:  
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(A) LENGTH: 20 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
40 (ix) SEQUENCE DESCRIPTION: SEQ ID NO:10:  
ACATGGGGGC ATCAGCAGAG 20

- 5 (2) INFORMATION FOR SEQ ID NO:11:  
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(A) LENGTH: 24 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
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(A) LENGTH: 24 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
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AGCTGACTCA GCCCTGCAAA GGCA 24
- 20 (2) INFORMATION FOR SEQ ID NO:13:  
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(A) LENGTH: 24 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
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TTATTAATTC CATTCAAAAT CATC 24
- 25 (2) INFORMATION FOR SEQ ID NO:14  
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30 (D) TOPOLOGY: linear  
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We claim:

1. A method for quantitation of RNA transcripts which comprises:
  - 2 a) extracting nucleic acid from a biological specimen;
  - 4 b) designing primer and probe sequences for amplifying mRNA and DNA by means of the same primer recognition sequences;
  - 6 c) hybridizing and amplifying said nucleic acid and said primer and probe sequences; and
  - d) quantitatively determining gene expression.
2. The method of claim 1 wherein said nucleic acid is a transcribed gene in which both the RNA and DNA sequences are characterized and possess sequence heterogeneity.
3. The method of claim 1 wherein said amplification of DNA and RNA is a competitive reaction, said DNA being an endogenous quantitation standard.
4. The method of claim 1 wherein said detection probes are designed to measure DNA intron sequences relative to mRNA sequences at the splice junction.
5. The method of claim 1 wherein said DNA and mRNA are amplified using a single set of primers and a plurality of selectively labeled probes.
6. The method of claim 5 wherein said primers are located within two exons flanking an intron, one of said probes is hybridized to said mRNA splice junction and a different probe is hybridized to an intron sequence of said DNA.
7. The method of claim 5 wherein one or both of said amplification primers is chemically modified or labeled.

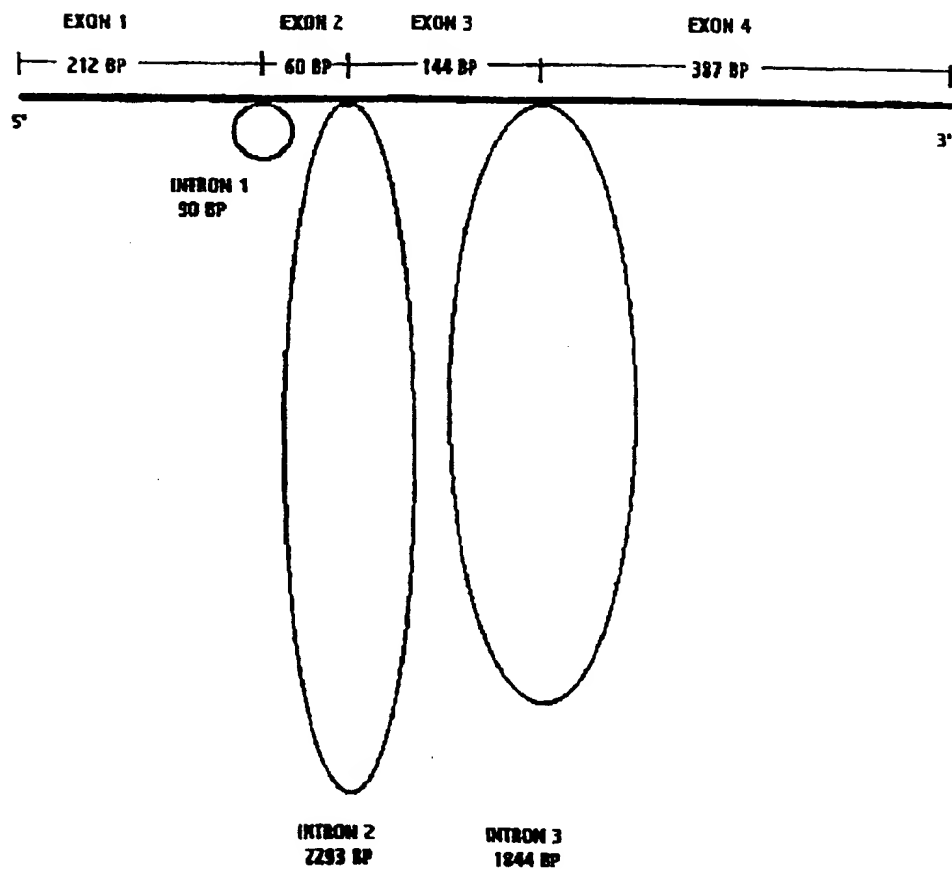


- 2 8. The method of claim 1 wherein said amplification is duplex amplification using a common primer capable of amplifying both genomic DNA and expressed mRNA from the same gene sequence.
- 2 9. The method of claim 8 wherein said primer detection probes are selectively labeled with or without a chemical modification to, or labeling of, the single common amplification primer.
- 2 10. The method of claim 8 further comprising a second DNA primer in the intron sequence and a second mRNA primer complimentary to the intron splice junction sequence, said second primers simultaneously acting as detection  
4 probes.
- 2 11. The method of claim 1 wherein said determination comprises the separation of amplimers by size.
- 2 12. The method of claim 11 wherein said separation comprises chromatography.
- 2 13. The method of claim 12 wherein said chromatography comprises gel electrophoresis, size-exclusion chromatography or high-performance liquid chromatography (HPLC).
- 2 14. The method of claim 1 wherein said probes are labeled with reporter molecules or haptens.
- 2 15. The method of claim 1 wherein said determination comprises sequence discrimination.

16. A method for quantitation of RNA transcripts which comprises:
- 2 a) extracting nucleic acid from a biological specimen;
  - b) designing primer and probe sequences for amplifying mRNA and
  - 4 DNA by means of the same primer recognition sequences;
  - c) performing PCR using a single tube, one step reaction wherein a
  - 6 distinguishable fluorescent signals specific to RNA or DNA are generated; and
  - d) determining relative gene expression through multiplex
  - 8 componenting the ratio of fluorescent signals or determining absolute RNA
  - copy number relative to a standard curve based on known concentrations of
  - 10 purified nucleic acid for a gene.
17. A method for quantitation of RNA transcripts which comprises:
- 2 a) extracting nucleic acid from a biological specimen;
  - b) designing primer and probe sequences for amplifying mRNA and
  - 4 DNA by means of the same primer recognition sequences;
  - c) amplifying nucleic acid via primer-based amplification
  - 6 methodology; and
  - d) specifically hybridizing and quantifying DNA and mRNA
  - 8 amplimers using labeled capture probes.
18. The method of claim 17 further comprising detecting capture probes
- 2 using a specific label on one or both amplification primers.

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# SCHEMATIC VIEW OF THE HUMAN IL-2 GENE:

**FIGURE 1**

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# SCHEMATIC VIEW OF THE OF THE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE GENE

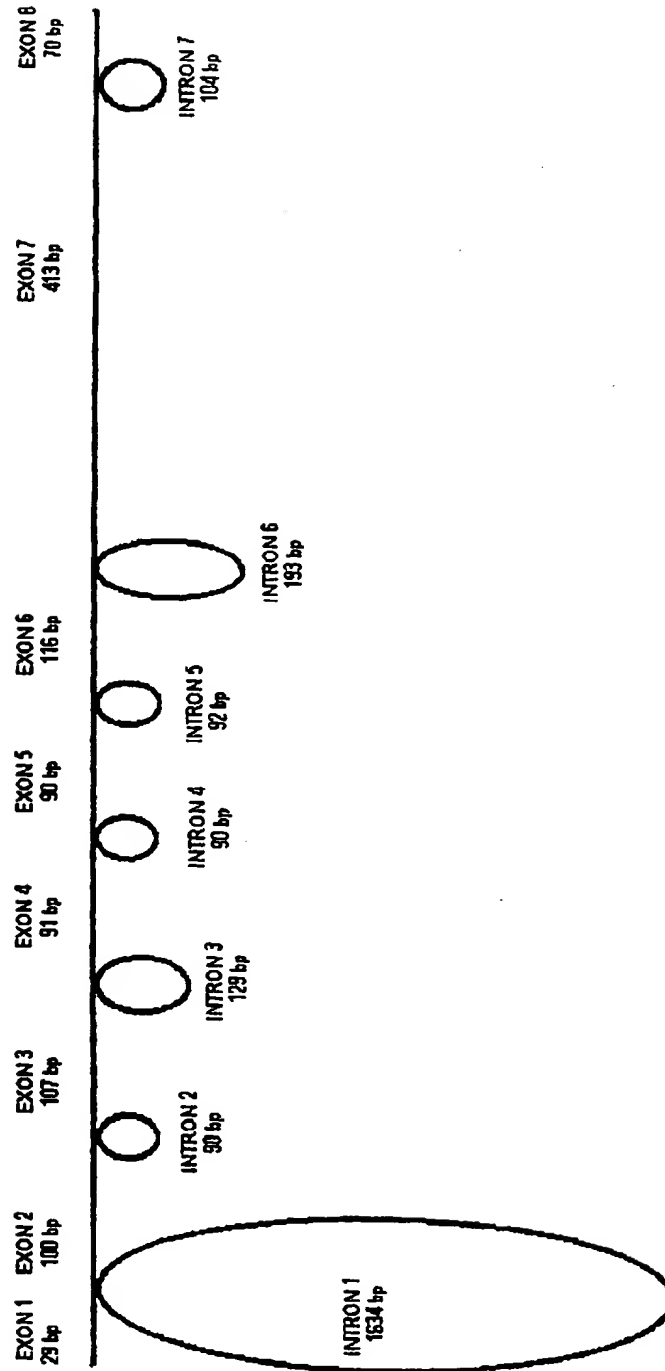
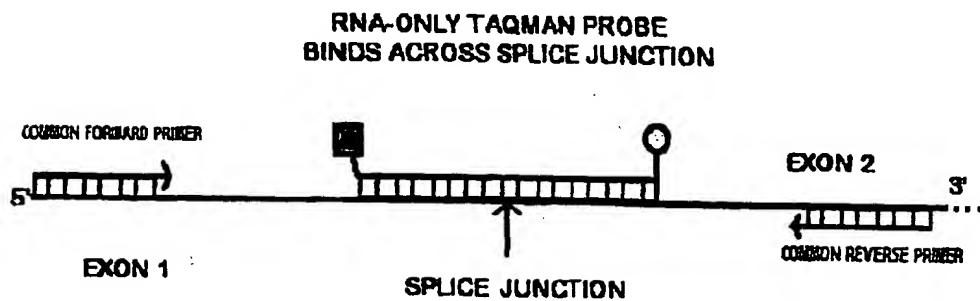
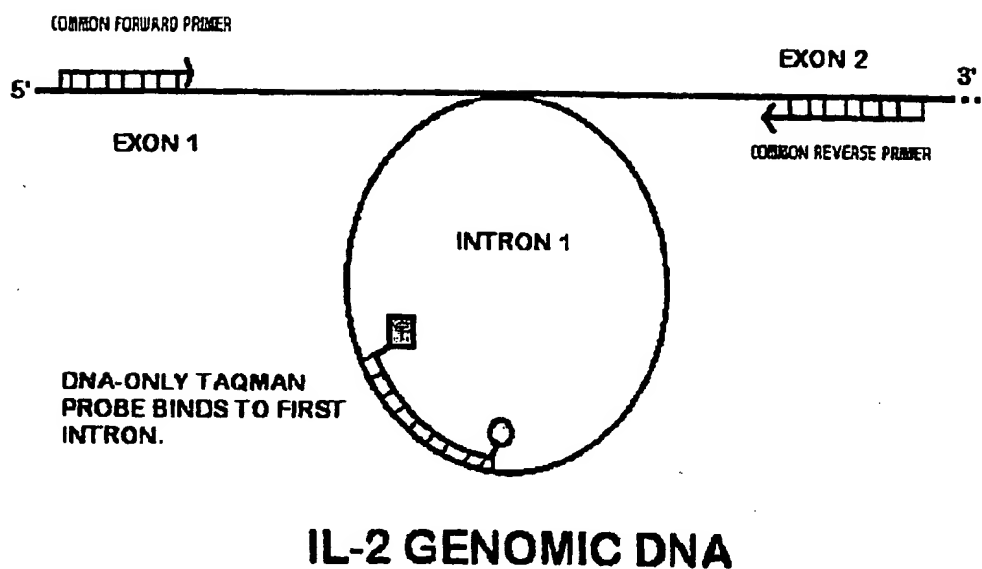


FIGURE 2

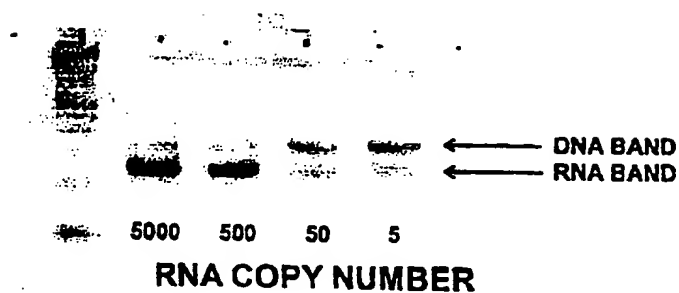
3/12  
**PROBE AND PRIMER DESIGN:**



**IL-2 mRNA**

**FIGURE 3**

### GAPDH PCR: mRNA TITRATION AT CONSTANT DNA CONCENTRATION



**FIGURE 4:** Agarose Gel Electrophoresis of 40-Cycle PCR amplification reactions. DNA copy number constant at 25 copies per reaction. Messenger RNA concentration varied from 5 to 5000 copies per reaction.

IL-2 INDUCTION: IL-2 SINGLE PROBE PCR  
RNA SIGNAL DURING INDUCTION PERIOD

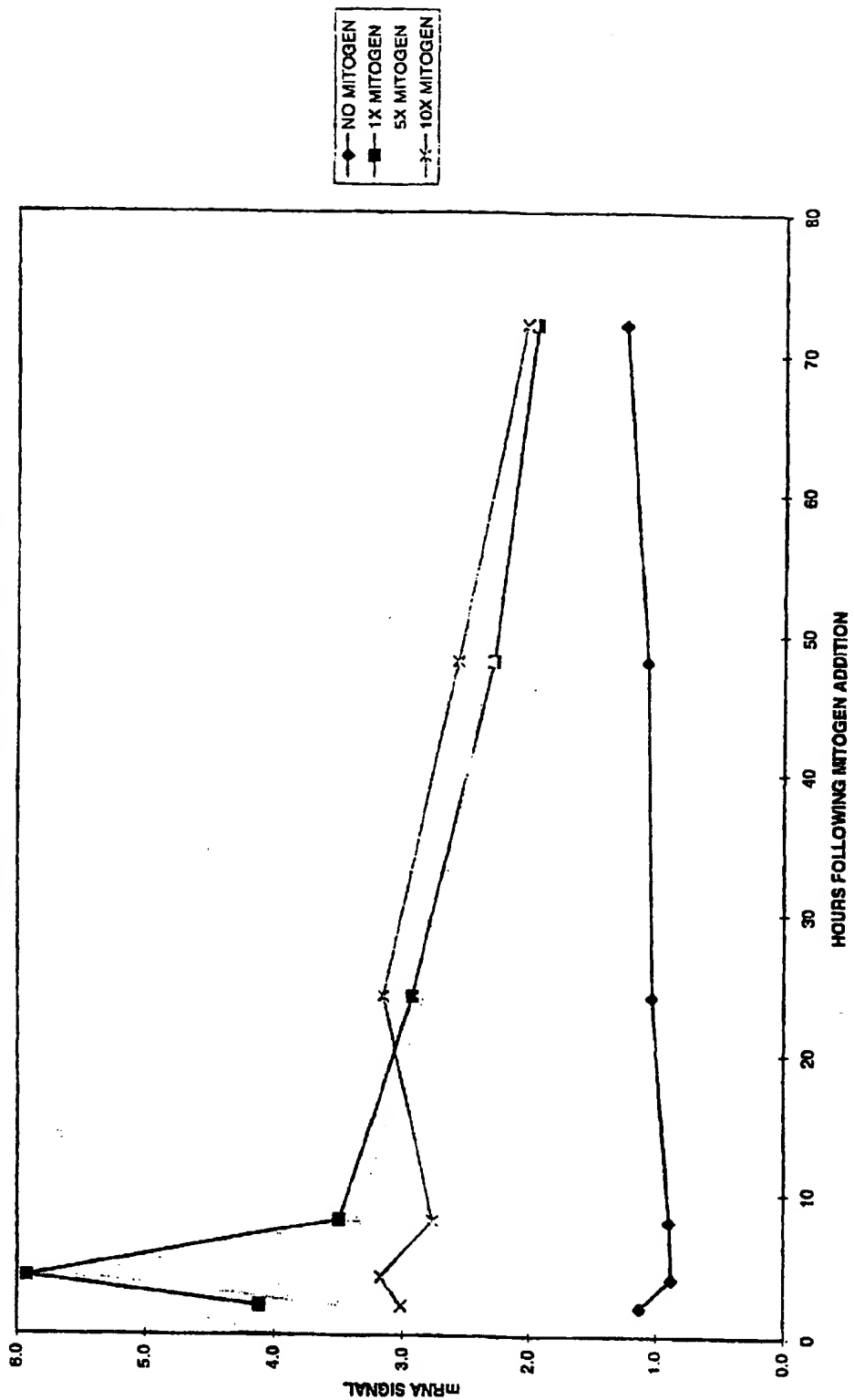
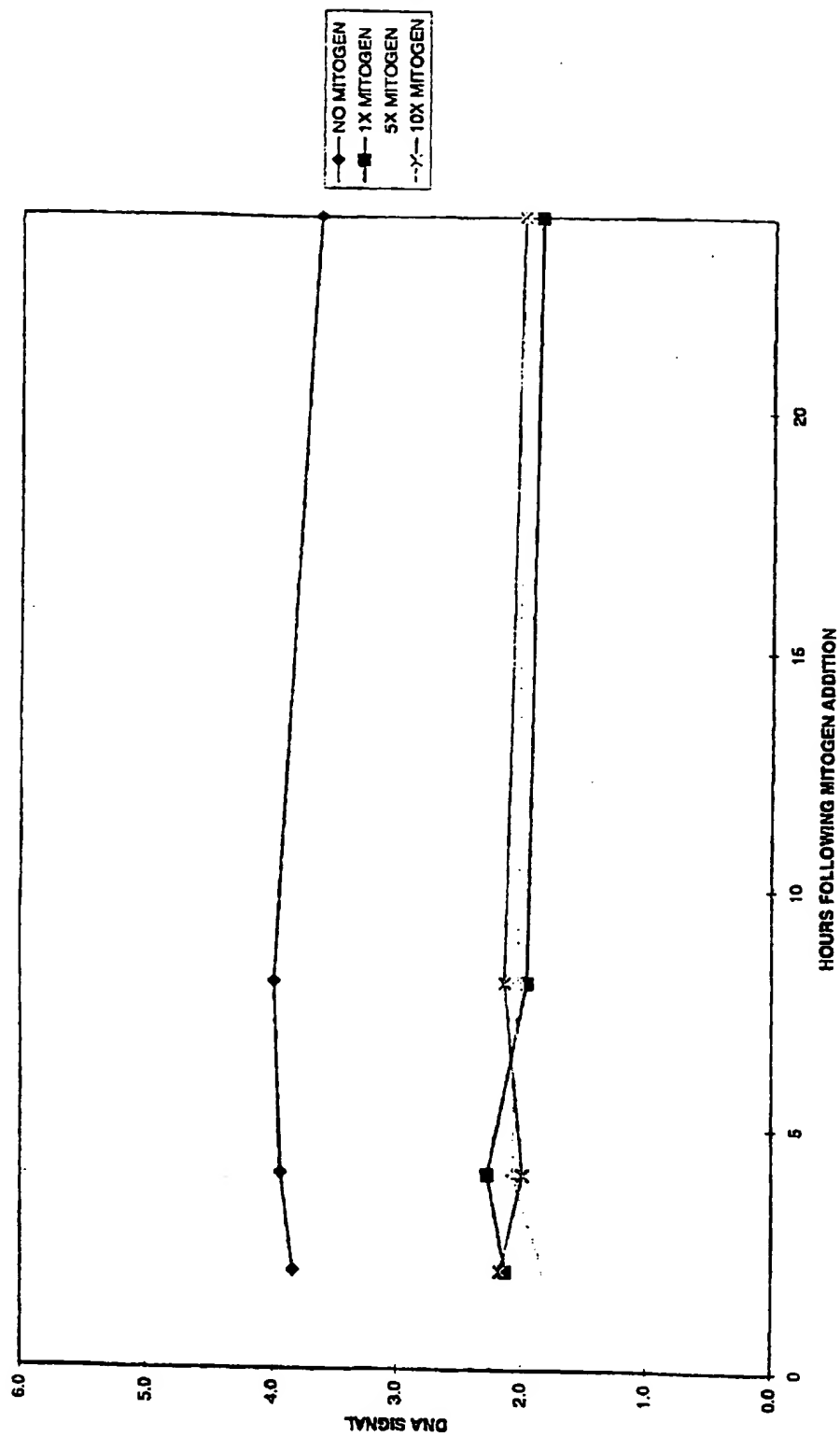


FIGURE 5

**IL-2 INDUCTION: IL-2 SINGLE-PROBE PCR**  
DNA SIGNAL DURING INDUCTION PERIOD



**FIGURE 6**



IL-2 INDUCTION: RATIO OF THE RNA/DNA SIGNALS  
FROM THE SINGLE-PROBE EXPERIMENTS

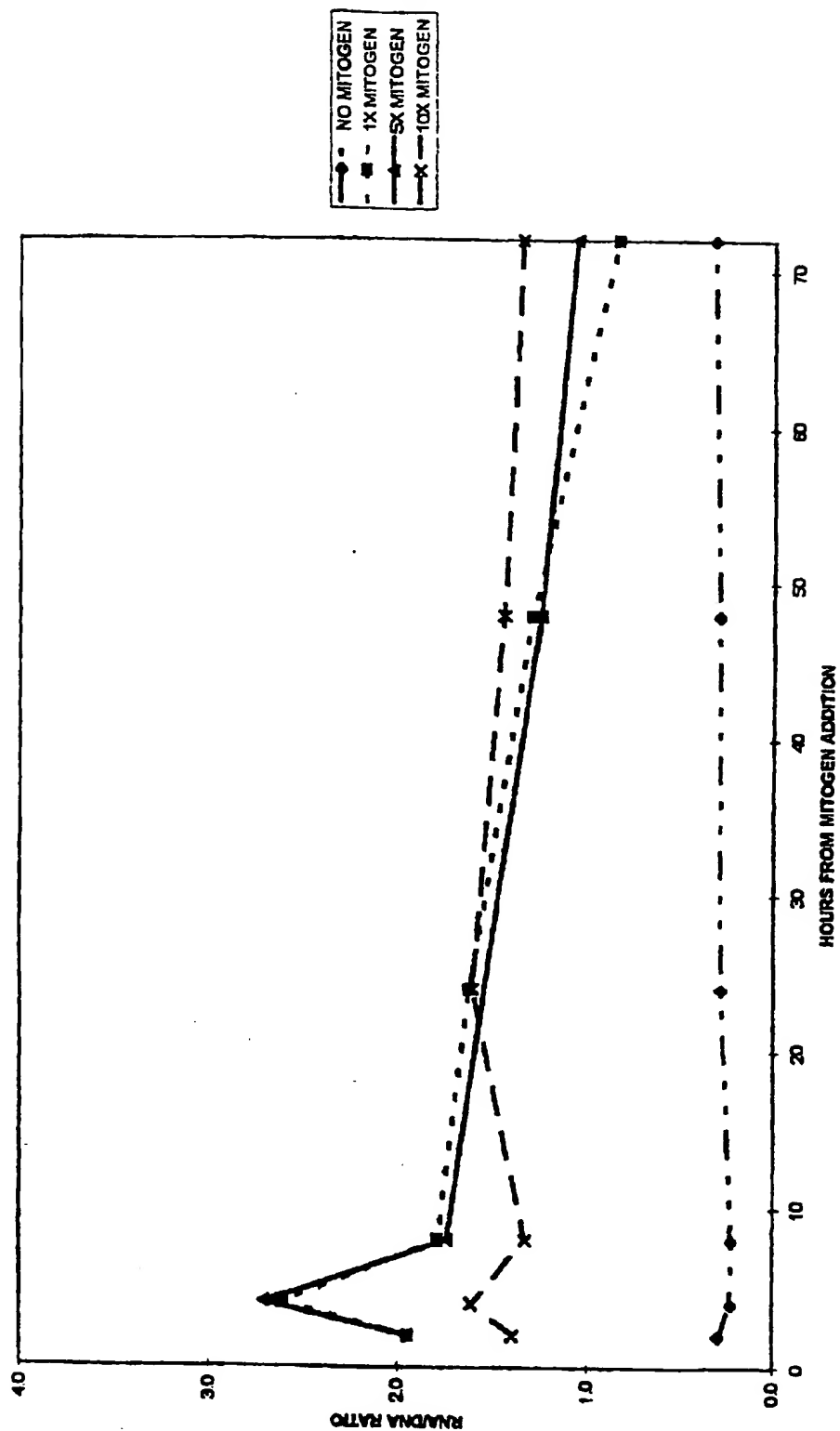


FIGURE 7

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# IL-2 mRNA INDUCTION DUAL PROBE TAQMAN PCR:

RNA:DNA SIGNAL RATIO VS. HOURS FOLLOWING INDUCTION

(Jurkat cell line, PHA, PMA mitogens)

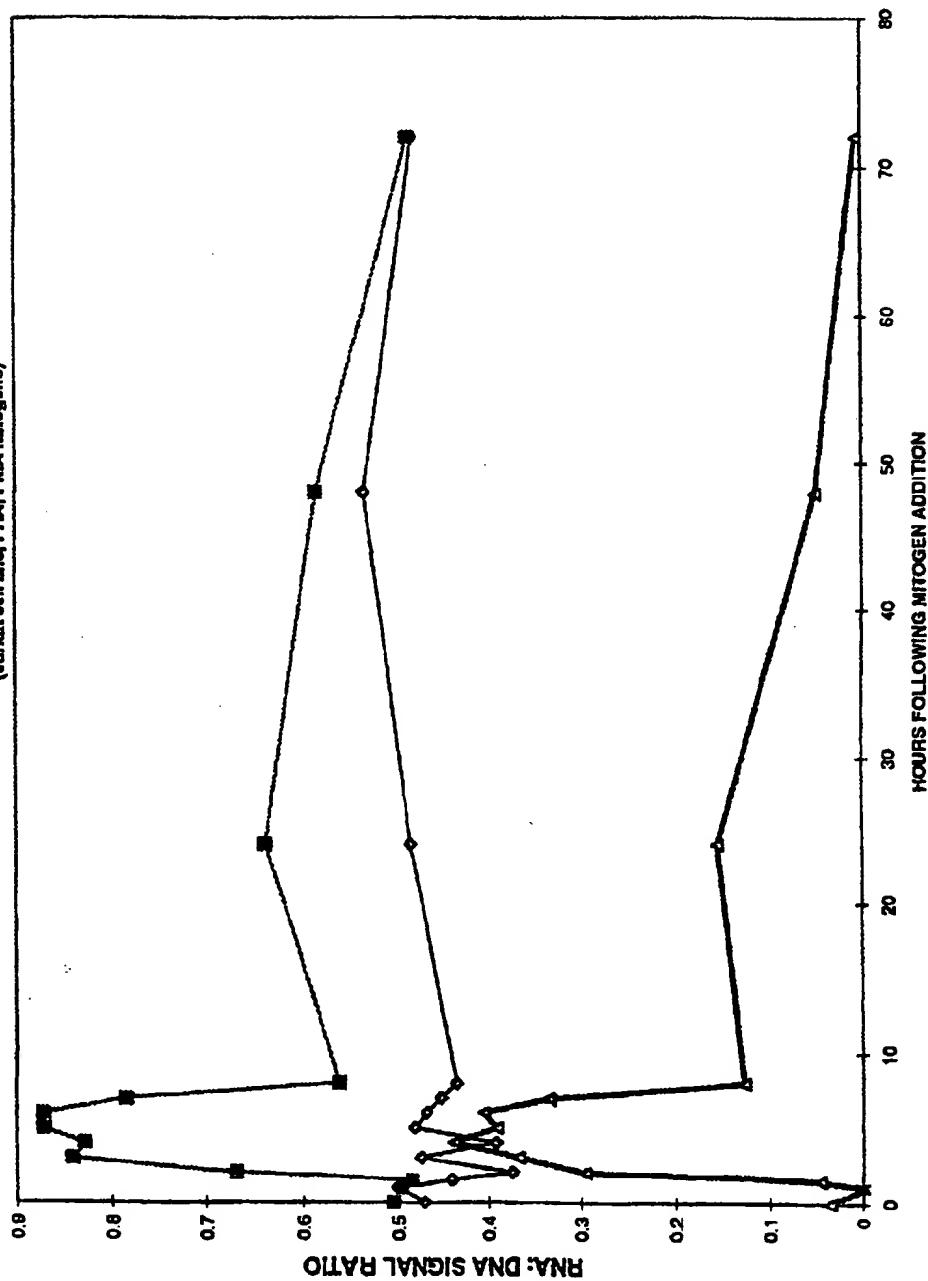
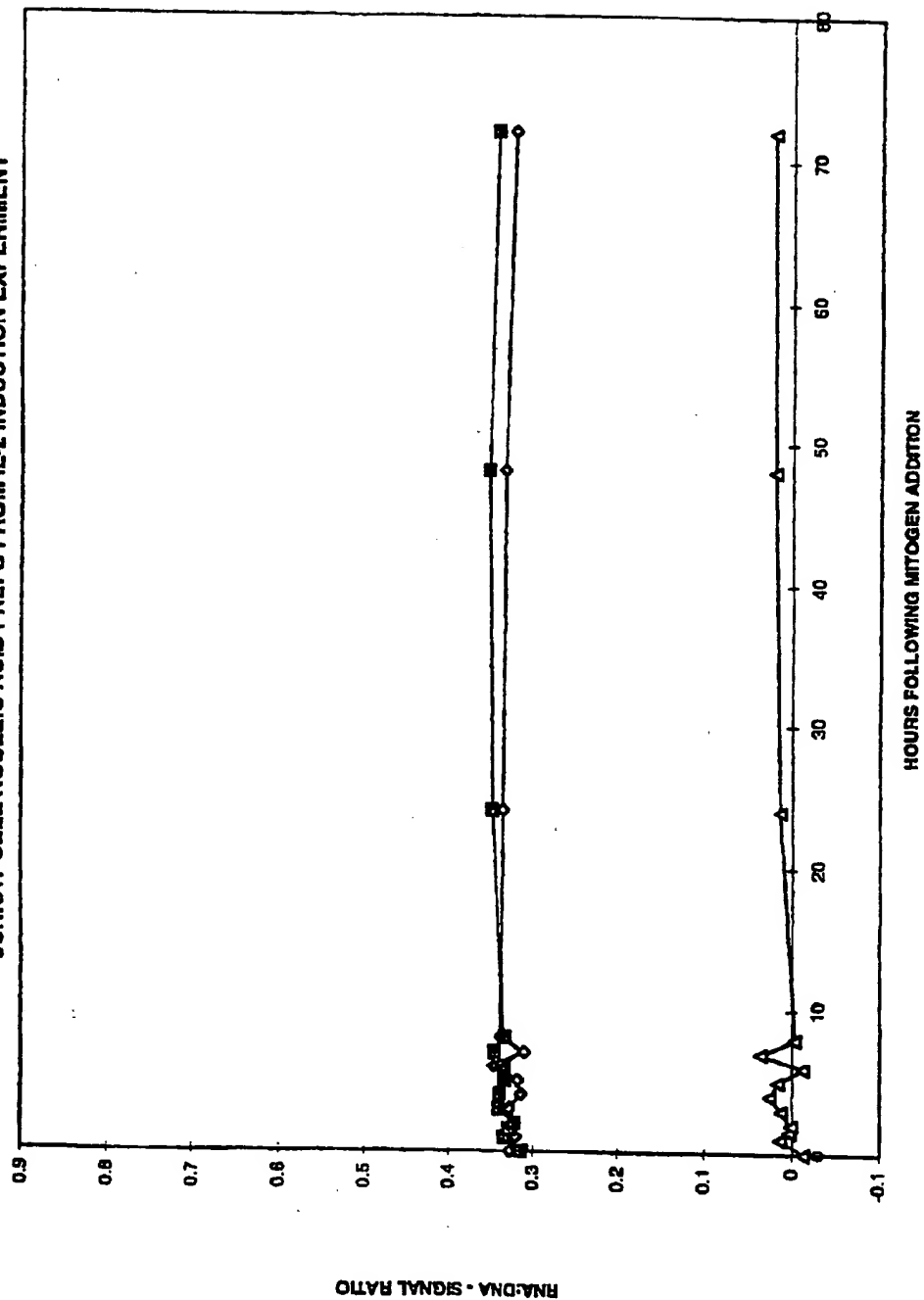


FIGURE 8

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**GAPDH RNA/DNA DUAL PROBE TAQMAN PCR**  
**JURKAT CELL NUCLEIC ACID PREPS FROM IL-2 INDUCTION EXPERIMENT**

**FIGURE 9**

## IL-2 PROTEIN EXPRESSION DURING INDUCTION PERIOD

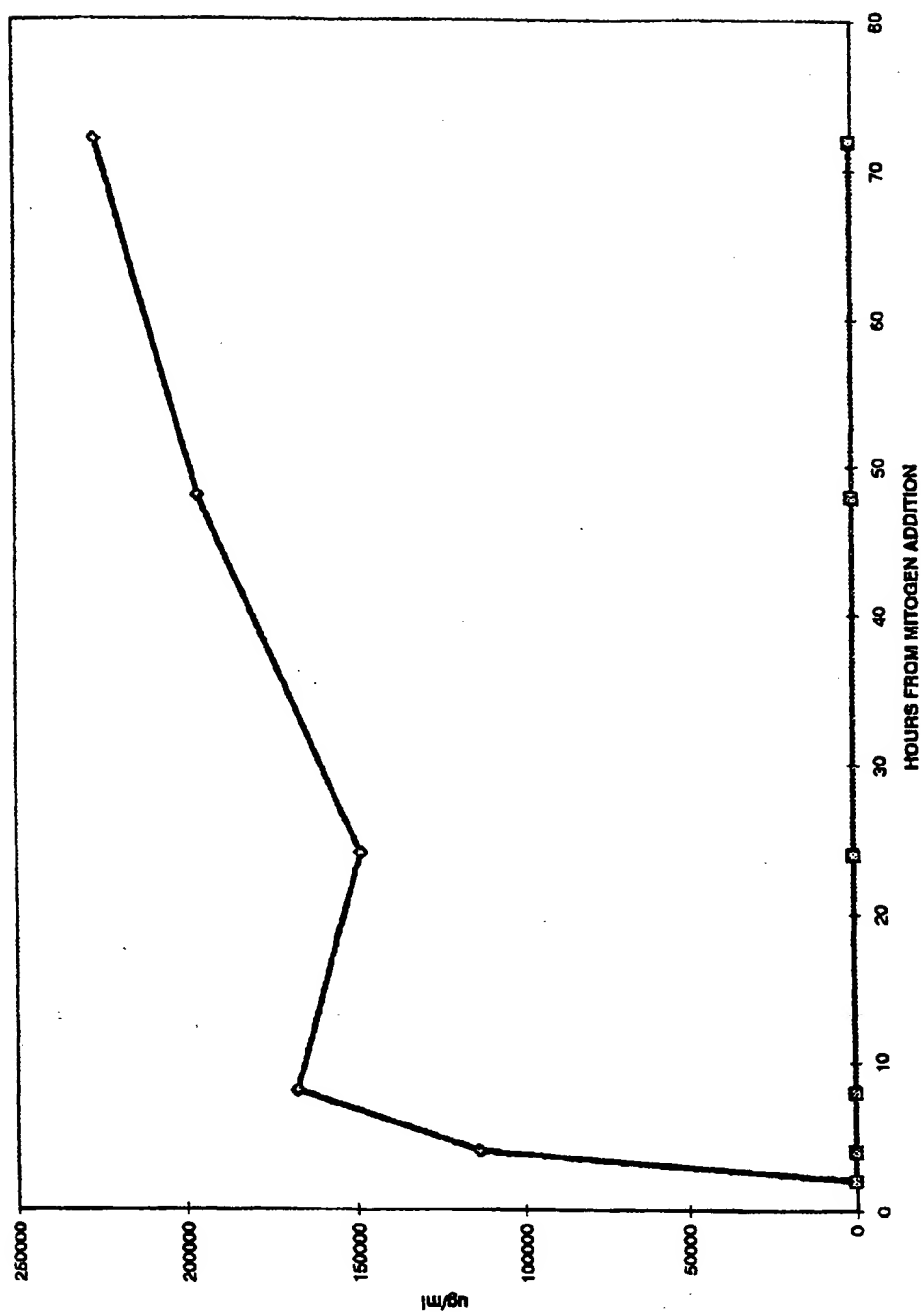


FIGURE 10

**Schematic Representation of Probing Scheme for Gene Expression  
Quantitation Using Splice Junction Probe for RNA detection, and Intron  
Probe for DNA Detection of Amplimers from Common Primers**

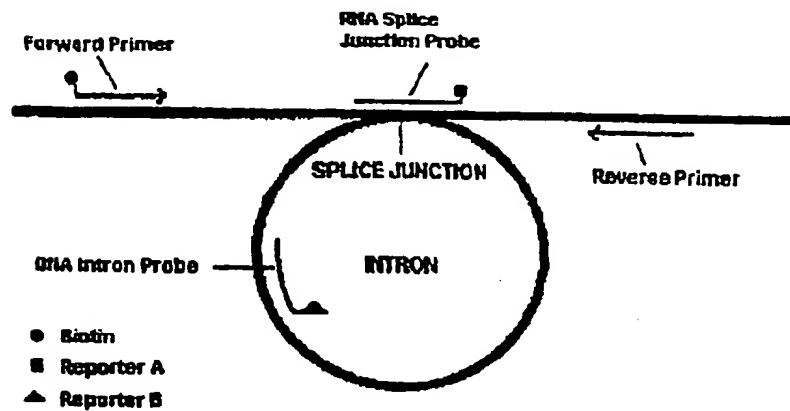
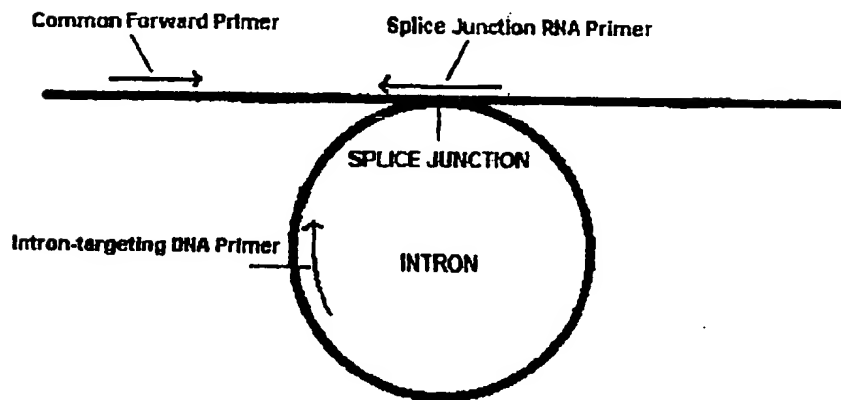


FIGURE 11

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Schematic View of Modified Primer Set where Reverse Primers are Discriminating primers for DNA and RNA amplifications.



The system would be used for  
 the system where there is a  
 one common primer set for  
 the forward primer and the reverse  
 primer and the intron is targeted.  
 This could be used if the intron were not by

FIGURE 12

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/07386

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12Q 1/68; C12P 19/34

US CL : 435/6, 91.2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2, 91.5; 536/24.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,213,961A (BUNN et al.) 25 May 1993, see entire document, especially abstract, Figure 1 and columns 9-11.	1-18
X --- Y	ZENILMAN et al. Competitive Reverse-Transcriptase Polymerase Chain Reaction without an Artificial Internal Standard. Analytical Biochemistry. January 1995, Vol. 224, No. 1, pages 339-346, see entire document, especially page 339-340 and Figure 1.	1-3, 5-6, 8-14, 16 ----- 4, 7, 15, 17-18
Y	JONES et al. RNA Isolation and Analysis. Oxford: BIOS Scientific Publishers Ltd. 1994, pages 15-93 & 129-143, especially pages 140-141.	1-18

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search

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Date of mailing of the international search report

26 SEP 1997

 Name and mailing address of the ISA/US  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

DEBRA SHOEMAKER

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/07386

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	US 5,580,970 A (HENDRICKS et al.) 03 December 1996, see entire document.	1-18



**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US97/07386

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

Searched inventors and keywords quantitate or quantify with mRNA or RNA or transcripts and splice junction or splice or junction with probe or primer and internal control or competitor in the databases APS, CAPLUS, MEDLINE, SCISEARCH, BIOSIS and WPIDS.